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Studijní program: Speciální chemicko-biologické obory:
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Bc. Petra Hadlová

Antigenom definuje výběr mutovaných peptidů

pro nádorově-specifickou T-buněčnou imunitní odpověď

Antigenome defines a selection of mutated peptides

driving tumor-specific T cell immune response

Bakalářská práce

Školitel: RNDr. Karel Drbal, Ph.D.

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Poděkování:

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Prohlášení:

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V Praze, dne 18.8.2016

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Bc. Petra Hadlová

Abstract:

T cells, as an essential part of the adaptive immune system, play crucial role in eradication of tumor growth. T cells target, interact with and eventually annihilate the tumor cells in antigen-specific (Ag) manner. T cells interact with tumor cells via short epitopes bound to the major histocompatibility complex (MHC) molecules on the tumor cell surface. Tumor specific neoepitopes arise from random somatic mutations and constitute a part of the tumor antigenome. Antigenome comprises of two classes of antigens, tumor specific antigens (TSA) and tumor associated antigens (TAA). TSA are neoantigens carrying neoepitopes unique to each tumor. TAA are self-antigens presented by both tumor cells and non-transformed cells.

Each tumor cell is able to develop numerous ways to evade the immune system consisting of T cells, NK cells, macrophages and other mechanisms employed. Despite that immunotherapy has shown a great potential in personalized medicine. The stratification of responsive patients is essential for effective and durable management of therapy in clinical practice. Methods are employed, which study existing reactive T cell clones, somatic mutations present in each patient, role of somatic mutations in tumor development and present neoepitopes. All these patient-specific features facilitate effective stratification. Patients benefit from long-term pathology-free survival response and limited adverse side effects.

Key words: antigenome, T cell response, somatic mutation, MHC-restriction, tumor biomarker, tumor immunotherapy, patient stratification, personalized medicine

Abstrakt:

T buňky, nezastupitelná součást adaptivního imunitního systému, hrají důležitou roli při eradikaci růstu nádoru. T buňky antigenně-specificky cílí, zajišťují interakci a následně i likvidují nádorové buňky. Interakci T buněk s nádorovými buňkami zprostředkovávají krátké, nádorově specifické epitopy. Epitopy jsou T buňkám prezentovány na povrchu nádorových buněk navázané na molekuly hlavního histokompatibilního komplexu (MHC). Nádorově specifické epitopy vznikají v důsledku náhodných somatických mutací a jsou součástí nádorového antigenomu. Antigenom se skládá ze dvou skupin antigenů, nádorově specificických (TSA) a asociovaných s nádory (TAA). TSA jsou unikátní pro každý nádor zatímco TAA jsou prezentovány nádorovými i vlastními buňkami pacienta.

Nádorové buňky vyvinuly několik způsobů jak imunitní reakci na úrovni T buněk, NK buněk, makrofágů i dalších mechanismů uniknout. Navzdory tomu, má imunoterapie v personalizované medicíně obrovský potenciál. Stratifikace reagujících pacientů je pro nasazení vhodné léčbu zcela zásadní. Využití spektra metod umožnilo studium mutací u jednotlivých pacientů, role mutací ve vývoji nádoru, existujících reaktivních T buněčných klonů a přítomných specifických epitopů. Tyto poznatky pomáhají navrhnout nejefektivnější stratifikace a eventuálně i léčbu zacílenou na nádor. Imunoterapie vede k dlouhodobé remisi a zároveň omezuje negativní dopady dosud používaných metod.

Klíčová slova: antigenom, T-buněčná odpověď, somatická mutace, MHC-restrikce, nádorový biomarker, imunoterapie nádorů, stratifikace pacientů, personalizovaná medicína

Abbreviations:

Ab	antibody
Ag	antigen
AML	acute myeloid leukemia
APC	antigen presenting cell
BCR	B cell receptor
CDR	complementarity determining region
CLL	chronic lymphocytic leukemia
CRC	colorectal carcinoma
ELISPOT	Enzyme-Linked ImmunoSpot
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MSI	microsatellite instable colorectal carcinoma
MSS	microsatellite stable colorectal carcinoma
NK cell	natural killer cell
NSCLC	non-small cell lung cancer
PBMC	peripheral blood mononuclear cell
pMHC	peptide-major histocompatibility complex
RCC	renal cell carcinoma
TAA	tumor associated antigen
T_{reg}	regulatory T cells
TCR	T cell receptor
TIL	tumor infiltrating lymphocyte
TSA	tumor specific antigen
wt	wild type

Content:

1 Introduction

T cells play a key role in eradication of tumor cells in the body. They interact via their T cell receptors (TCR) with the epitopes, antigenic determinants, presented on tumor cell surface. High mutation rates within the tumor lead to the production of *de novo* expressed antigenic epitopes, which make the tumor cell a suitable target for the T cell adaptive immune response. Despite the high efficacy of adaptive immune response, tumors evade the immune response by continuous random generation of escape clonal variants. The evasion mechanism poses a challenge for the complete, durable eradication of tumor cells.

Tumor cells are also capable of recruiting normal cells to create an ideal microenvironment for the tumor growth. Tumor microenvironment suppresses the immune response (1). Recruited cells present commonly occurring epitopes on their surface. This leads to weakened immune recognition of the tumor mass because the recruited cells are recognized as self. The creation of tumor microenvironment both protects the tumor cells from the immune response and provides nutrients and oxygen for the tumor cells. Nonetheless a T cell immune response is capable of targeting the tumor cells precisely and eradicate them efficiently via their specific neoepitopes. The aim of this thesis is to present the current state of research on the subject of tumor antigenome-T cell interaction, its potential for patient stratification and the closely connected personalized medicine.

2 The role of T cells in tumor-targeted immune response

2.1 T cell development

T cells are central organizing elements of adaptive immune response. T cells are of hematopoietic origin and their repertoire is generated during T cell differentiation in the thymus by mechanism of central tolerance. T cells acquire specific recognition potential exclusively to their own MHC alleles in the thymic cortex. This recognition limitation is termed MHC restriction and it occurs during the process called negative selection. In the process of negative selection, the non-reactive T cells are annihilated. However self-peptides bound to MHC molecules must not be recognized by T cells. Therefore, self-reactive T cells are usually also obliterated in the process of negative selection in the thymic medulla. As a result, T cells

generally do not react against the cells of their own body. Nonetheless, a minimal autoreactivity (towards self-epitopes) is still part of normal peripheral T cell repertoire. To ensure development of reactive T cell clones, positive selection leads to survival of such T cells that are capable of interaction with self MHC molecules. The mechanisms of positive and negative selection are presented in *Fig.1* (2).

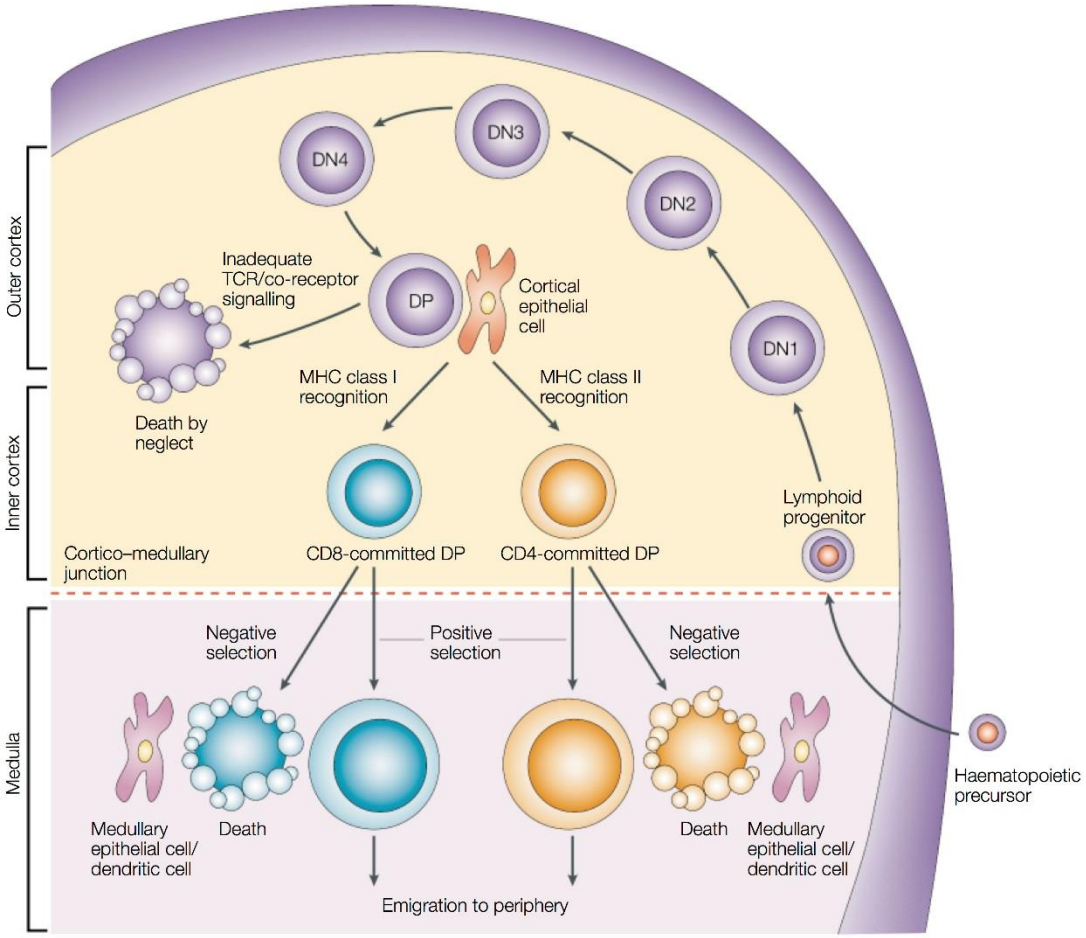

Fig.1 ***Thymic processes of central tolerance.*** *The progenitor lymphoid cells are produced in the bone marrow and they migrate to the thymus. In the thymus they are exposed to numerous signals. In the early stages of development, thymocytes do not express TCR, CD4 and CD8 co-receptors and therefore they are called double-negative thymocytes (DN1-DN4). During their maturation process thymocytes express pre-TCR after successful TCR β (δ) VDJ rearrangement and selection. If pre-TCR signaling occurs, immature thymocytes rearrange the second TCR gene (α, β) and massively proliferate during the transition from DN 4 to double-positive (DP). The DP thymocytes are contacted with self MHC class I and MHC class II molecules by cortical thymic epithelial cells presenting self-peptides. The further fate of thymocytes is determined by their signaling in response to the interaction with self-peptide MHC (pMHC) complexes. Too little signaling by T cell leads to death by neglect (delayed) allowing for positive selection in the cortex. Too strong signaling leads to acute apoptosis during the negative selection in the medulla.* (2)

After thymic emigration the process of T cell activation is completed on the periphery in three steps. The first step of the activation of naïve T cells is enabling an efficient adaptive immune response against any novel Ag. Such Ag is in most cases distinct from any self-structure since self-reactive T cells have been eliminated in the thymus. Second key objective is differentiation to effector T cells in several polarized lineages. Third the process must generate memory T cells, which allow the organism to react much faster if re-challenged with exactly the same epitope (2).

As shown in *Fig. 2* T cells are only a part of a vast range of immune cells contributing to the defense against development of tumor including both innate and adaptive immunity. All immune cells involved are part of the so called extrinsic tumor-suppressor mechanisms. Extrinsic tumor-suppressor mechanisms comprise the molecular mechanisms of non-transformed cells used to detect the presence of transformed tumor cells and restrict growth of the tumor mass (3). The individual mechanisms of the interaction of immune system with the tumor mass are depicted in *Fig.2*. Interaction between tumor and adaptive immune system develops in three phases: elimination, equilibrium and escape (4).

The immune system also prevents the development of tumor cells in three ways. First it protects the organism by eliminating viral infections, a mechanism which facilitates effective prevention of virally induced tumors. Second, elimination of other pathogens prevents the development of chronic inflammation. Chronic inflammation frequently contributes to tumorigenesis so by preventing it immune system prevents tumor development. Third specific targeting of tumor cells by immune system in a process called immune surveillance (4). Immune surveillance keeps the tumor growth under control. Alongside the T cells, other immune cells, such as NK cells, B cells, macrophages and dendritic cells play an important role in immune surveillance.

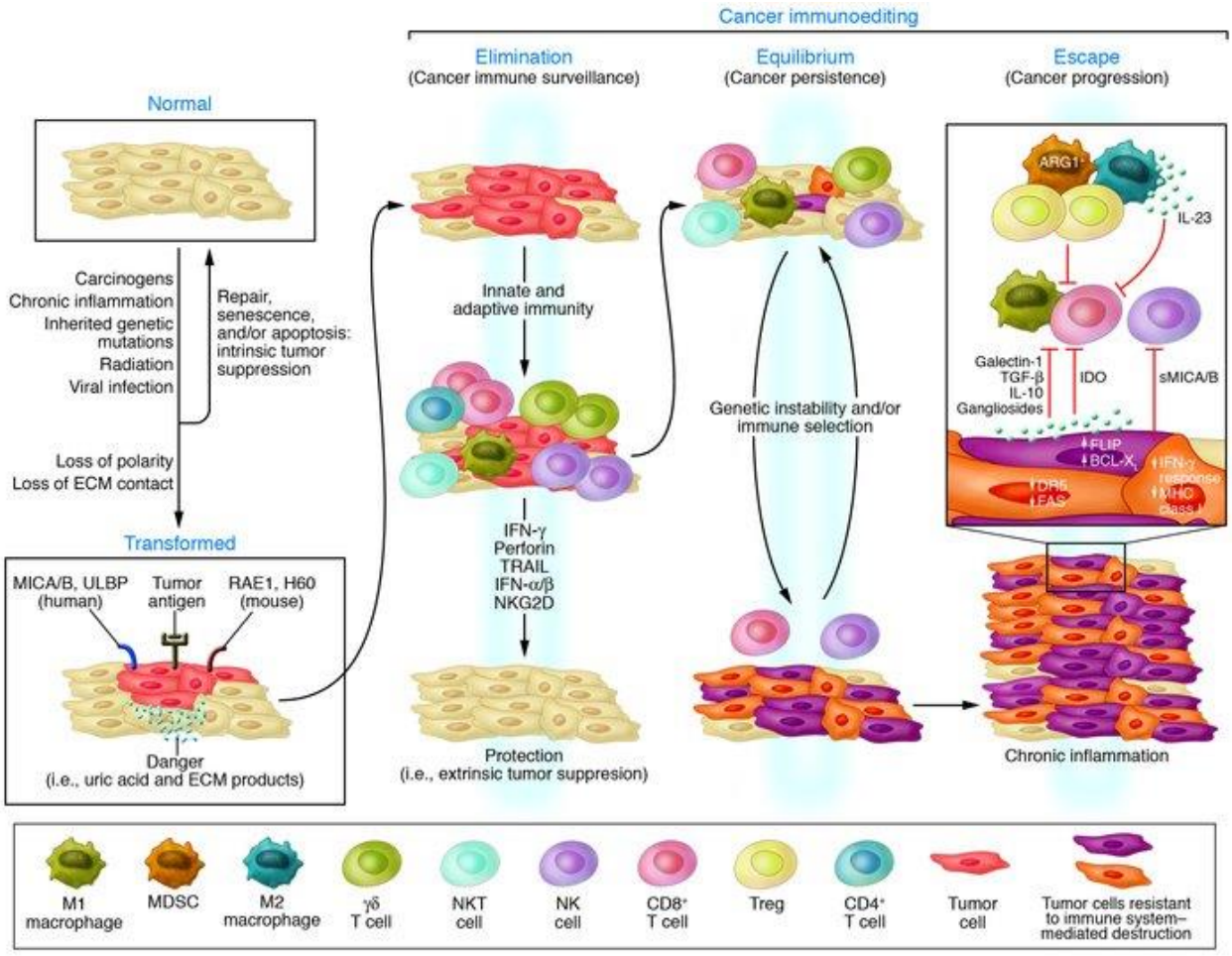

Fig. 2 ***<u>Interaction between tumor cells and immune system.</u>*** *Figure 2 shows an overview of the immune cells involved in eradication of tumor cells and the mechanism of escape from the immune response. Elimination is a step in which tumor has been successfully eradicated mainly by the cells of the adaptive immune system, predominantly T cells. Equilibrium is a stage in which the tumor growth is kept under control by the immune system, but is already present in a dormant, non-progressive form. The escape phase is the phase in which cells of the adaptive immune response are no longer capable of keeping all de novo generated tumor escape clones under control – the tumor diversifies and develops into a larger tumor mass and eventually metastasizes* (4).

2.2 Epitope

Interaction of T cells with other cells is organized in an Ag-specific manner. As evident from *Fig.1*, T cell function depends on the interaction between T cells and MHC class I and class II molecules. T cells target tumor cells via binding to their epitopes presented on MHC molecules. Epitope is the Ag determinant recognized by an antibody, mediated by B cell receptor (BCR) or via variable CDR loops of TCR. A depiction of epitope binding to MHC and TCR is shown in *Fig. 3*. The antigenicity of the peptides is what is relevant to the thesis. Antigenicity is the ability of the epitopes to bind to TCR with optimal affinity and induce T cell adaptive immune response. Interaction between epitope and TCR occurs after APC presents the epitope in form of peptide-MHC complex (pMHC) to a reactive T cell clone together with appropriate costimulation (5). T cell pool responds effectively by clonal expansion of particular Ag-specific T cell clones.

Activated T cells bind antigenic epitopes directly on tumor cell surface already without costimulation and cause tumor eradication. High diversity of T cell clones guarantees high specificity of interaction with almost any peptide. A specific reactive T cell clone is able to recognize a very delicate sequential (linear) change, which is conveyed to a single amino acid exchange in the mutated neoepitope (2). T cells are predetermined target the newly arising amino acid sequence motifs (neoepitopes) efficiently. Structural epitopes are recognized by B cells.

The efficiency of the T cell reactivity is subsequently enhanced in the process of continuous T cell clonal expansion of reactive T cell clones. The clonal expansion of T cells provides a strong immune response, which enables tumor regression (6). Importantly for the patient stratification, the binding affinity of neoepitopes to MHC molecules is predictable. On the other hand, the binding affinity of the pMHC complex to TCR is random and can only be tested later indirectly by T cell reactivity.

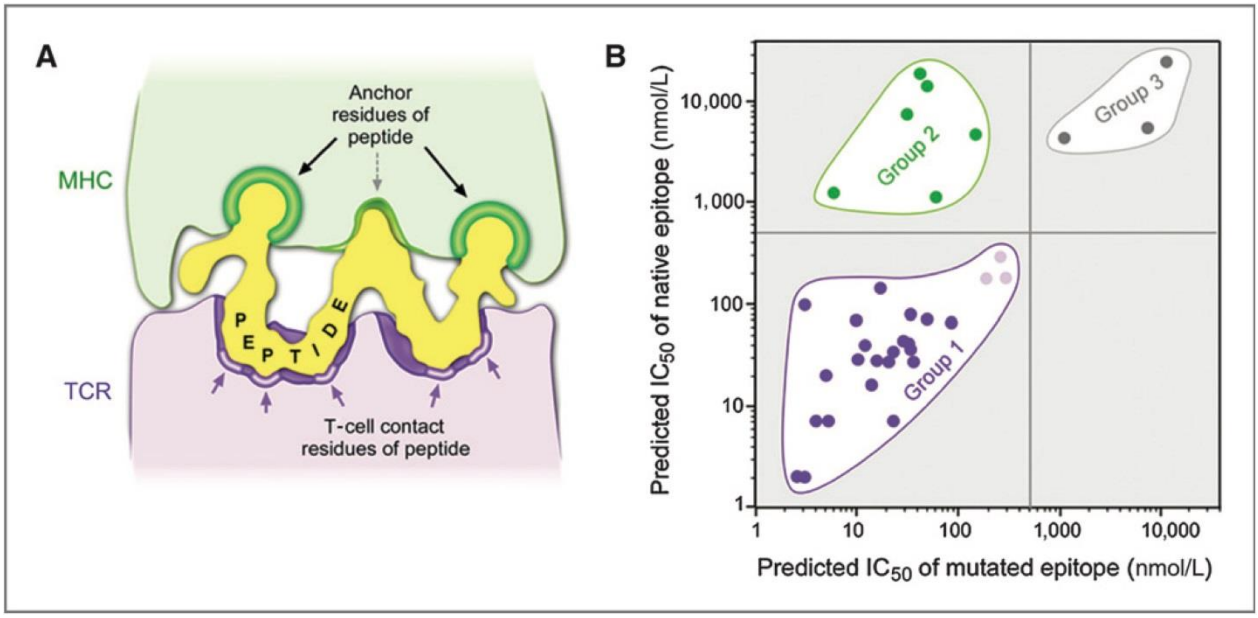

Fig.3 (A) ***Antigenic peptide binding to MHC and TCR.*** *The two faces of a bound peptide to the MHC and TCR molecules form a "double-sided key", such structure is necessary to stimulate an Ag-specific immune response. Green color indicates the anchor amino acid residues, which interact with MHC molecules and purple is used for the part of peptide that interacts with TCR surface, namely the CDR loops. (B)* ***Binding affinities of epitopes to MHC molecules.*** *A scatterplot of the affinities of epitopes that stimulate detectable T cell responses is shown. Group 1 are epitopes with comparable predicted affinities of native and mutated epitopes. Group 1 has mutations in peptide regions critical for interactions with TCR, dark purple areas are strong and light purple are weak binders. Group 2 are epitopes with strong predicted affinity whose corresponding native peptides are not predicted to bind MHC molecules and they have mutations in peptide residues crucial for the interaction with MHC molecules. Group 3 are epitopes in which neither native nor mutated peptides are predicted to be MHC binders* (5).

2.2.1 Role of somatic mutations in defining neoepitopes

The tumor-specific epitopes recognized by T cells are quantitatively and/or qualitatively different from their normal tissue counterparts. The tumor epitopes may be divided into two groups. First group are the rather nonspecific self-epitopes or tumor associated antigens (TAA). These are presented in different quality/quantity by tumor cell from those presented by normal tissue. Second group of tumor epitopes and mutations encoded epitopes (neoepitopes) also termed tumor specific antigens (TSA). Neoepitopes are derived *de novo* from the DNA somatic mutations in tumor cells (7). There is a great variability in the literature concerning terminology related to these two groups of tumor epitopes. The self-epitopes presented by both normal and tumor cells, are termed TAA or self-antigens (6). As for the neoepitopes they are termed TSA (8, 9), or neoantigens (6, 10–12). For the purposes of this thesis the newly arising peptides will be termed neoepitopes. Neoepitopes are the most specific part of the neoantigen, in which the most

delicate mutational changes are detectable by the TCR. Peptides presented by both normal and tumor cells will be termed self-epitopes.

Self-epitopes will be introduced only very briefly and will not be the main subject of this thesis. Nonetheless they are significant. They can cause autoreactivity leading to autoimmune diseases in patients. This problem arises because tumors are in principle self-structures and autoreactivity is a normal feature of our immune reactivity. Regarding immune response to tumor, autoreactivity is enhanced in patients after immunotherapy. Self-epitopes belong to four major groups:

- mutation antigens, which are antigens arising from specific mutations or translocations
- tumor-germline or the so-called cancer-testis/oncofetal antigens, which are not or only weakly expressed by the normal adult tissues and re-expressed by tumor cells
- overexpressed antigens, which are normally present in non-transformed tissue but highly upregulated in tumor cells
- differentiation antigens, presenting a pattern that is restricted to defined differentiated cell types (13) usually different from the tumor cell type.

Neoepitopes, on the other hand, are largely patient-specific or rather tumor-specific and they are strongly antigenic. Neoepitopes are caused by random somatic mutations during tumor development. Neoepitopes are not tolerated by the adaptive immune system as shown in *Fig.4* (6). While the self-epitopes are present in a large spectrum of patients, they do not induce such a strong adaptive immune response. Lack of immune response is due to established central tolerance. Despite all the counterarguments a certain level of autoreactivity is desirable during immunotherapy. This has been proved by the compilation of a list of 230 self-epitopes specific for melanoma, which could serve as a database for future analysis before patient stratification (13). The autoreactivity could increase the efficiency and success of the applied treatment leading to complete and durable tumor regression.

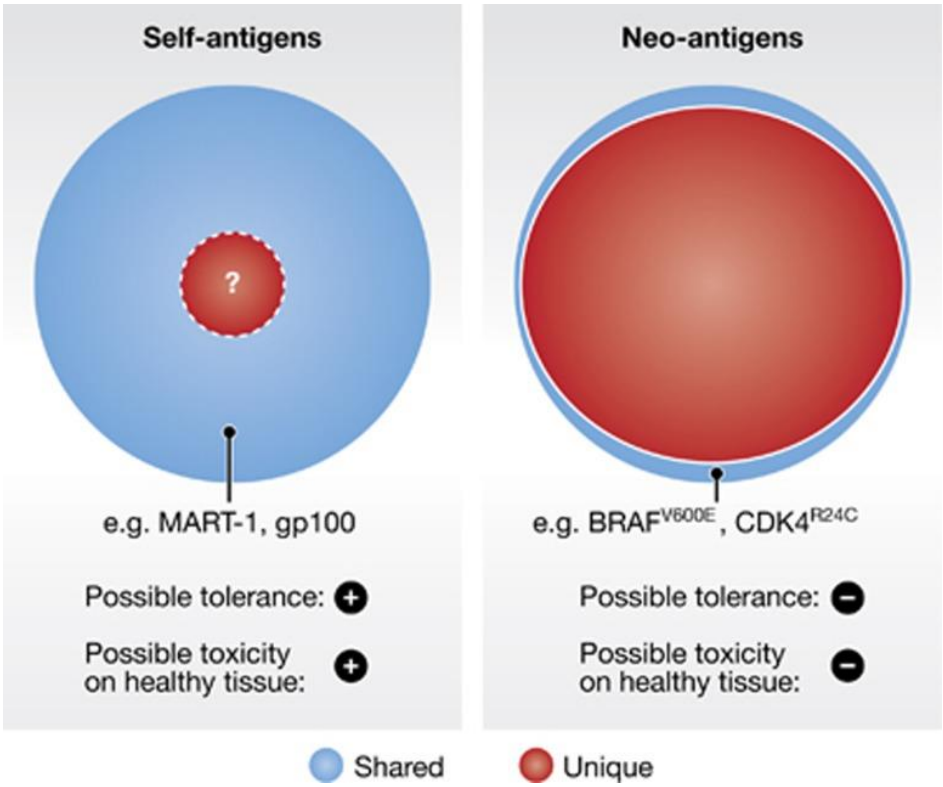

Fig. 4 ***Representation of distribution of self- versus neo- epitopes and their characteristics regarding tolerance and toxicity for the body.*** *Blue color indicates the self-epitopes, shared among many patients and red circle indicates patient specific neoepitopes. As depicted in this figure the self-epitopes are not in most cases specific for a single patient and they are tolerated by the immune system. Neoepitopes are not tolerated and as evident from the description there is no damage done to the healthy tissue when neoepitopes are targeted by the adaptive immune system* (6).

The more mutations encoding neoepitopes occur in the tumor, the higher the chance for pre-existence of a specific, reactive T cell clone and consequently the better prognosis for the patient. The set of mutations leading to tumor development, metastases or appearance of treatment-resistant clones serve as tumor-specific part of exome. That is a minor subset of cancer genome, specific to each individual tumor or even a part of tumor mass (14).

2.3 Peptide presentation to T cells

Peptides, arising from the mutations in the tumor are presented to T cells and consequently exposed to T cell immune response. The mechanism of peptide presentation must be described in order to understand the significance of T cell adaptive immune response in tumor eradication. Peptides are loaded onto MHC class I molecule in the endoplasmic reticulum and endosomes. During the process of peptide loading onto the MHC molecule a competition occurs among the peptides generating selection of the peptide with optimal binding affinity (15). To identify the peptides with optimal binding affinities, which are most likely to bind to the MHC molecule,

potential 8-11mer peptide-MHC pairs have been evaluated and analyzed (16). Optimal binding affinity was shown to be < 50nM (15, 17). In some peptides only the mutant form shows high binding affinity (15). A database has been created predicting the MHC binding peptides. The catalogue identifies neoepitopes with high binding affinity resulting from point mutations (18, 19). The binding affinity of a peptide to MHC proteins strongly corresponds to the antigenicity of such peptide (20, 15).

2.4 Role of T cells in immune response to neoepitopes

T cells are responsible for Ag-specific eradication of diverse agents in the body including tumor cells (11). Naïve peripheral T cells are well equipped to identify large MHC peptides presented on the surface of cells and react towards such cells accordingly. CD8+ T cells recognize MHC class I bound epitopes and are capable of efficiently eradicating the tumor mass without disturbing the surrounding tissue the way traditional, commonly applied therapies do. The T cell clone reactivity is one of the most accurate prognostic factors. For successful application of T cell oriented immunotherapy, it is absolutely essential for the patient to have pre-existing reactive T cell clones. The application of methods, predictive of the patient´s reaction to therapy has been used by research teams (11, 21, 22) which led to successful employment of the T cell immune response.

There is a great heterogeneity of T cells present in the renal cell carcinomas (RCC). Only 0.24-16.82% of T cell clones were detected in all regions of the RCC, from which the samples were extracted. It has been determined that the composition of the rest of the T cell subpopulations is heterogeneous across different regions of the same RCC. The heterogeneity has been studied by the ultra-deep TCR-sequencing technology with the depth of up to several hundred thousand reads. Unique T cell clones were detected in thousands of reads, from 3780 to 25930 (23) as shown in *Table 1*. Understanding the heterogeneity as well as the clonality of the T cell repertoire is essential for the identification of responsive patients, who would achieve highly specific durable eradication of the RCC. T cell clone reactivity of the particular patient can be detected by its cognate epitopes if present within the RCC (23). T cells are capable of producing multiple clonotypes specific for the same mutant epitope (24). Additionally they are able to target multiple neoepitopes simultaneously (8). These two traits enable efficient eradication of the tumor cells.

Intratumour heterogeneity of T cell clones

	Analysis based on all detected T cell clones/tumour				Analysis based on the 100 most frequent T cell clones/tumour			
	RMH002	RMH004	EV003	EV004	RMH002	RMH004	EV003	EV004
Unique T cell clones	25 930	25 471	8809	3780		N/A		
Proportion of ubiquitous clones (%)	16.82	1.31	0.45	0.24	97.00	67.00	16.00	7.00
Proportion of heterogeneous clones (%)	83.18	98.69	99.55	99.76	3.00	33.00	84.00	93.00

Table 1 ***Intratumor heterogeneity of T cell clones in RCC****. The table shows the proportion of unique T cell clones compared with the vast majority of heterogeneous clones as found in a study conducted by* (23)*. The codes of numbers and letters are identifiers of individual patients.*

A great advantage is that the reactive T cell clones, which are present in a patient can be sorted and expanded *in vitro* by a factor of up to 1000 fold. After *in vitro* expansion they are reapplied into the patient, in a procedure called adoptive T cell transfer (25, 26). A great advantage of this method is that even T cells isolated from peripheral blood can undergo the *in vitro* clonal expansion. This method is minimally invasive, which makes it highly attractive for research as well as clinical application. The T cells are analyzed and sorted using flow cytometry (24, 25).

To identify the reactive T cells in the clinic ELISPOT was used. It is a method, which detects the IFNγ secretion (27). T cell populations responding to the cognate epitope secrete high levels of IFNγ and do not secrete any in response to the self-epitopes (24). Interestingly this method served Kreiter and his team to confirm the presence of MHC class II molecules. MHC class II molecules indicate the presence of CD4+ T cells at the tumor site. Generally, CD8+ T cells are accepted as the most strongly correlated with patient survival (28). Presence of CD4+ reactive T cell clones was confirmed by another research team (29). Using a murine model, the researchers have proved that majority of antigenic neoepitopes was indeed recognized by CD4+ T cells. Researchers claim that up to 80% of neoepitope-targeted immune response was imposed by CD4+ T cells (27). The MHC class II molecules, in contrast to MHC class I molecules (binding 8-11 mers) (16), bind 11-20 amino acid long peptides of extracellular origin. They present an additional pool of potential prediction markers, since they induce such a massive T cell response (30). These are significant findings, because an exploitation of such massive pool of possible targets could serve for further improvement of personalized immunotherapy. However the currently available prediction programs for binding affinities of neoepitopes to MHC class II are not yet accurate enough to serve the purpose as explained below (30).

2.5 Tumor infiltrating lymphocytes

Highly important population of T cells regarding the stratification of patients are tumor infiltrating lymphocytes (TIL). TIL frequently infiltrate the tumor site and have both positive and negative functions. Presence of TIL at the tumor site indicates good prognosis for the patient based on data collected from six tumor sites (28). Therefore, they are another candidate predictive marker. In some cases, TIL are capable of mediating durable regression of metastatic melanoma, lasting more than 9 years (25). TIL represent an excellent target for further research into the personalized therapy since they provide a collection of patient-specific cells, which are reactive towards tumor neoepitopes (31). They are also thought to facilitate the identification of tumor reactive T cell clones (29). It has been observed that TIL are abundant, they are present in 96,6% of colorectal carcinomas (CRC) and 70% out of 598 genomic datasets of CRC show a T cell phenotype (32).

The limitation of exploitation of TIL is the frequency of neoepitope-reactive T cell clones in TIL bulk populations. The low frequency might be overcome by the use of MHC class I tetramers generated with candidate mutant epitopes previously identified by whole-exome sequencing combined with prediction methods and followed by tetramer staining (24). Despite the rather rare occurrence of neoepitope-reactive T cells among TIL, researchers were able to identify markers, which indicate the ability to eradicate tumor cells. The most abundant markers are inhibitory receptors PD-1 *(programmed cell death protein-1)*, LAG-3 *(lymphocyte activating gene 3)*, and TIM-3 (*T cell immunoglobulin and mucin domain containing protein 3*). Their expression is being researched as another suitable marker for the set of patient-specific, tumor reactive CD8+ T cells (33). Cohorts of the neoepitope-reactive T cells were shown to express high levels of PD-1, which makes them suitable targets for the PD-1 checkpoint inhibitor therapy (10, 11). For a cohort of patients with PD-L1 *(programmed cell death ligand-1)* expression in NSCLC when they presented high rates of non-synonymous mutations, up to 91% experienced durable clinical benefit (34). Nonetheless it is important to keep in mind that those patients´ NSCLC were largely PD-L1 positive.

Additional factor, which serves as an indicator of reactivity of TIL is a co-stimulatory 4-1 BB. As shown in *Fig.5* the PD-1 and 4-1 BB markers are specific candidates for prediction of responsiveness to treatment in oncological patients (31). SPAG5 *(sperm associated antigen 5)* and TSSK6 *(testis specific serine kinase 6)* were identified as putative antigenic cancer/testis epitopes in multiple tumors.

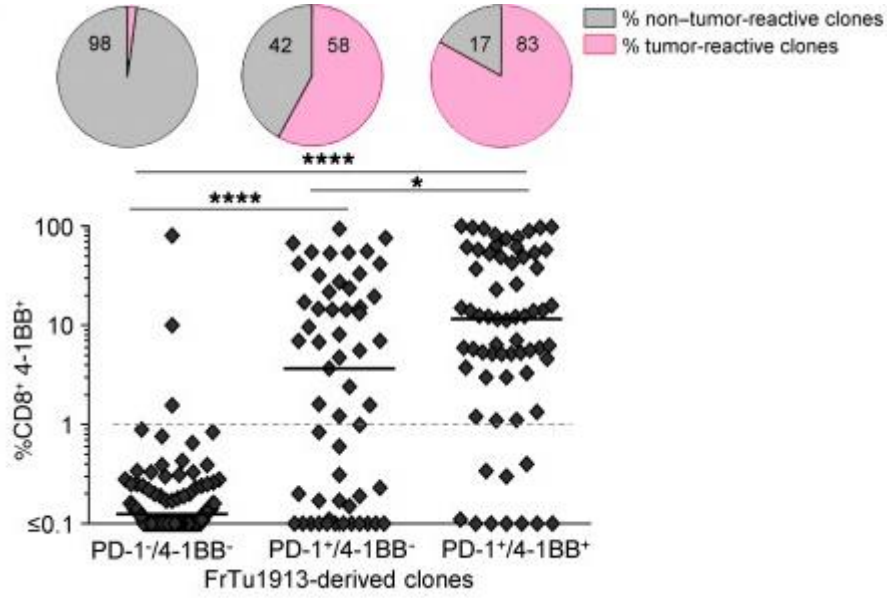

Fig. 5 ***Relevance of PD-1 and 4-1 BB markers in identification of reactive TIL in autologous tumor cell line TC1913.*** *Depiction of an experiment, in which cells derived from fresh tumor were responding to TC, autologous tumor cell line. TIL were divided into three groups, PD-1$^-$/4-1BB$^-$, PD-1$^+$/4-1BB$^-$ and PD-1$^+$/4-1BB$^+$. 4-1BB upregulation, which correlate with tumor responsiveness of a particular clone. The pie charts show the percentage of tumor-responsive (pink) and tumor non-responsive (grey) TIL clones for each population* (31).

No significant difference was observed in the patients with reactive TIL regarding sex, age, MHC type or metastatic stage (35–38). Therefore, these characteristics do not serve as a robust prognostic tool. From the intrinsic characteristics of the responsive TIL populations, the most important are features of TIL telomeres. Telomeres were longer in patients with present reactive TIL clones, which makes them a yet another suitable target for prognosis. Higher numbers of CD8+CD27+ memory T cells and higher numbers of *in vivo* persisting administered cells in the circulation (39) were also indicative of better prognosis. It was observed that CD27 contributed to Ag-specific expansion of T cells, which explains why it serves as a predictive marker (40).

Computational method was developed to infer the complementarity-determining region 3 (CDR3) sequences of TIL in 9,142 RNA-seq samples across 29 tumor types. Over 600,000 CDR3 sequences were identified, including 15% that were full length. TIL CDR3 sequence length distribution and amino acid conservation in many tumors, except brain and kidney tumors, resembled CDR3 from healthy donors' peripheral blood mononuclear cells (PBMC). A strong association between T cell diversity and tumor mutation load was observed. Finally, three potential antigenic somatic mutations were identified on the basis of their co-occurrence with CDR3 sequences. One of them, a PRAMEF4 *(PRAME family member 4)* mutation encoding p.Phe300Val, was predicted to result in peptide binding strongly to both MHC class I and class II molecules. Contemporary analyses have the potential to simultaneously identify antigenic neoepitopes and tumor-reactive T cell clonotypes (16).

2.6 Experimental methods

In order to understand how the information on the T cells and neoepitopes are gathered in research, several methods must be introduced. There are two major groups of methods used in the research of the immune response to cancer growth. These are predictive and experimental methods.

2.6.1 Predictive methods

The predictive methods, are predominantly *in silico* methods. Predictive algorithms identify candidate neoepitopes and estimate their antigenicity based on their predicted binding affinity to MHC molecules. The binding affinity is assessed based on the data collected for each individual patient (30). An overview of the most commonly used predictive methods is presented in *Table 2*. NetMHC algorithm is one of the most frequently used predictive methods regarding binding affinities in oncology research today (5, 16, 17, 29, 30). It is used for prediction of both MHC class I and class II molecules. For MHC class I molecules NetMHC provides the best data for both *in vivo* and *in vitro* application. NetMHC algorithm provides the most accurate data for MHC class II prediction as well. The only disadvantage of the NetMHC is that the rare alleles are more difficult to predict due to lack of *in vitro* data (30).

Name	**Validated *in vitro***	**Pros**	**Cons**	**MHC class prediction**
BIMAS	Yes	Used historically	Predictions for selected MHC A, B, C alleles	I
RANKPEP	Yes	Widely validated	Predictions for selected MHC A, B, C DP, DQ, DR alleles	I, II
SYFPEITHI	Yes	Widely validated	Predictions for selected MHC A, B, C alleles, class II limited to DRB1	I, II
NetMHC pan and NetMHC II pan 3.0	Yes	Pan-specific, class I best in vivo and in vitro data, class II predictor most accurate	Uncommon alleles more difficult to predict due to limited in vitro data	I, II
IEDB	No	Combines multiple predictive algorithms	Not yet validated	I, II
TEPITOPE	Yes	Pan-specific, widely validated	Probably not as efficient as NetMHC pan 2:0	II
Pickpocket	No	Pan-specific	Not available to public	II
MULTIPRED2	N/A	Pan-specific, combination of other programs	Not yet validated	II
MultiRTA	No	Built exclusively on experimental data	Not yet validated	II

Table 2. Overview of predictive MHC binding algorithms *The most effective programs for MHC class I prediction are the BIMAS, SYFPEITHI, RANKPEP and Net MHC. The high polymorphism of MHC II and flanking residues lead to MHC class II predictors underperforming MHC class I predictors. As a result, when searching for MHC class II prediction only, the TEPITOPE is used because it attempts to overcome the problem of lacking in vitro data* (30).

A procedure has been established in order to narrow down the vast pool of potential candidate neoepitopes. The procedure which identifies the neoepitopes to be considered for immunotherapy starts with *in silico* prediction. NetMHC algorithm reduces the number of selected peptides based on several criteria: predicted binding affinity, length of the predicted peptides and their expression levels (21, 25, 29). This method reveals the MHC class I binding molecules and allows further exploitation. Initial selection is paired with mass spectrometry, which enables the identification of MHC bound peptides from cultured cells (16). Predicted peptides are synthesized and tested (12). After application of the carefully selected cells the

results are verified by cDNA amplification and subsequent sequencing validates the whole exome sequencing results (22). Research proved this combination of predictive methods to be highly efficient and the results of one such experiment, performed on generated data, were relevant, as shown in *Fig.6*.

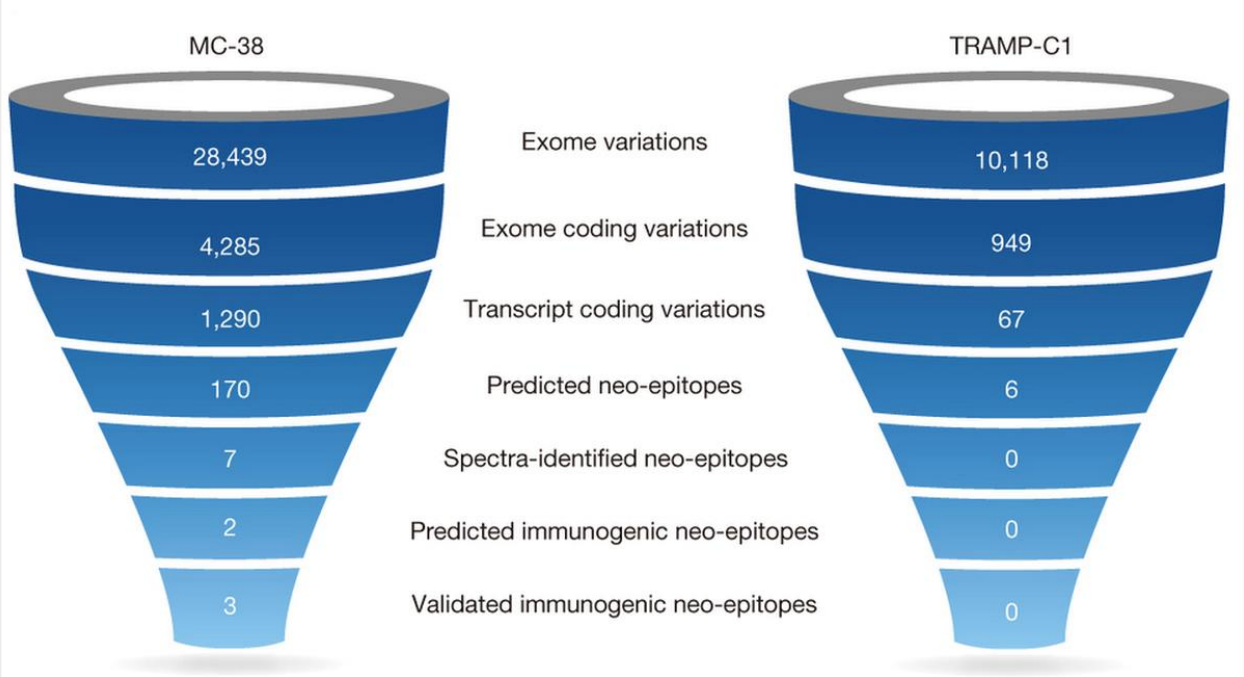

Fig.6 ***Prediction of antigenic neoepitopes****. As shown in this graphic the vast number of exome variations present in the beginning of the research has been narrowed down to reasonable 3 antigenic neoepitopes for MC-38, derived from murine colon adenocarcinoma and in the case of TRAMP-C1, transgenic murine epithelial prostate cell line, 0 candidate neoepitopes. In the case of TRAMP-C1 the result clarifies the low immunogenicity and antigenicity in vivo* (41).

2.6.2 Methods for detection of Ag-specific T cells

There are also experimental methods, which analyze and help understand specificities of T cell driven immunity in each individual patient. Flow cytometry, mass spectrometry and exome sequencing are among the most abundant. These methods are used to identify the reactive T cell clones in the patient as well as the candidate neoepitopes inducing reactivity. Each of the most frequently used methods will be shortly introduced.

Flow cytometry has been the leading method for quantitative measurement of cellular diversity in recent years. Cytometers can process up to tens of thousands of cells per second (42), analyzing vast cohorts of data. Flow cytometry enables single cell analysis based on the surface markers, providing statistically robust data for highly heterogeneous T cell pool. Flow cytometry

also enables detection and analysis of exceptional cell subpopulations. Currently used cytometers can sort cells based on one to two dozen parameters. Flow cytometry has been used to visualize both CD4+ (27) and CD8+ (10) reactive T cells in patient samples. T cells have been sorted using flow cytometry according to their reactivity to pre-selected MHC tetramers to identify the reactive T cell clones in each individual patient (24).

Another method used in oncological research is mass spectrometry (23, 5, 22). Mass spectrometry is often combined with exome sequencing to identify candidate neoepitopes. Mass spectrometry analyzes cell surface based on its chemical complexity (43). An outline of the process of mass spectrometry is presented in Figure 1 in a review by Römpp and Spengler (44). Data acquired from mass spectrometers contain information about spatial organization as well as mass spectral information (44).

Sequencing methods are used to identify the candidate target neoepitope sequences. Sequencing data help identify single point mutations, which can be highly antigenic and lead to substantial improvement of patient´s prognosis (28). To identify the genomic determinants of response to PD-1 blockade therapy in non-small cell lung carcinoma (NSCLC), whole-exome sequencing was employed. 94.5% of the candidate sequence were covered to the depth 10x. This experiment led to establishing the positive correlation between genomic landscape of lung tumors and response to anti-PD-1 therapy (34). In a different study whole-exome sequencing was used to identify non-synonymous mutations in DNA samples collected from 8 patients with metastatic melanoma (24).

A rather marginal method, ELISPOT, also proved useful in the research in the past. Today it is a rather surpassed method used in the clinical practice rather than in primary research. It has been replaced due to high background interference and compared to flow cytometry rather low statistical robustness. ELISPOT, with splenocytes that were cultured in an anti-IFNγ coated plates and the cytokine secretion was detected after overnight culture by anti-IFNγ antibody. ELISPOT is usually used to verify the presence of reactive T cell clones (27).

So far three predictive markers related to tumor-targeted T cell immune response have been described. These are the presence of TIL at tumor site, exploitations of present inhibitory receptors and MHC class II molecules. These markers serve as excellent prognostic tool. All of the above described methods are frequently used in research in order to identify more candidate neoepitopes and determine reactivity of present T cells. The reactivity of present T cells is

essential for good patient prognosis. Further research is needed to expand on the understanding of tumor-targeted T cell immune response. Nonetheless it has already been clearly proved that T cells are crucial in tumor eradication processes. T cells offer an effective immune response to tumor growth and have a great potential to be exploited in immunotherapy.

3 Role of mutations in tumor development

3.1 Tumor definition

Development of any spontaneous tumor is a result of a sequential series of alterations in well-defined genes altering the function of a limited number of pathways. The set of somatic mutations are caused by genome instability in the primary tumor cell mass. Somatic mutations accumulate within a small cluster of cells, the preneoplastic lesion which gives rise to the primary tumor or in tumor cells leading to metastases (45). This definition stresses the crucial role of somatic mutations in the development of tumor growth. On the other hand the tumor somatic mutations are also reservoirs of exploitable neoepitopes (6). Over the past decade the genomic landscape of common forms of human tumors has been revealed owing to the use of comprehensive whole-exome sequencing efforts (46).

3.1.1 Intrapatient differences in mutations

Types of tumor somatic mutations resulting in neoepitope presentation and subsequently T cell immune response range from a single nucleotide substitutions, small insertions and small deletions to copy number changes, DNA rearrangements and structural variants at the level of chromosomes (6, 26, 47). There are usually around 100 somatic mutations per tumor in most adult tumors (45). Typical rates of mutations for most commonly occurring tumors are shown in *Fig.7*. There are three main features, which determine the development of a tumor based on data collected for melanoma, bladder, cervical and ovarian tumors. The efficacy of T cell adaptive immune response targeting the tumor cells is influenced by: genetics of the tumor and its influence on the presentation of tumor neoepitopes, genetic characteristics of the patient, which influence the T cell repertoire and the environmental factors (48).

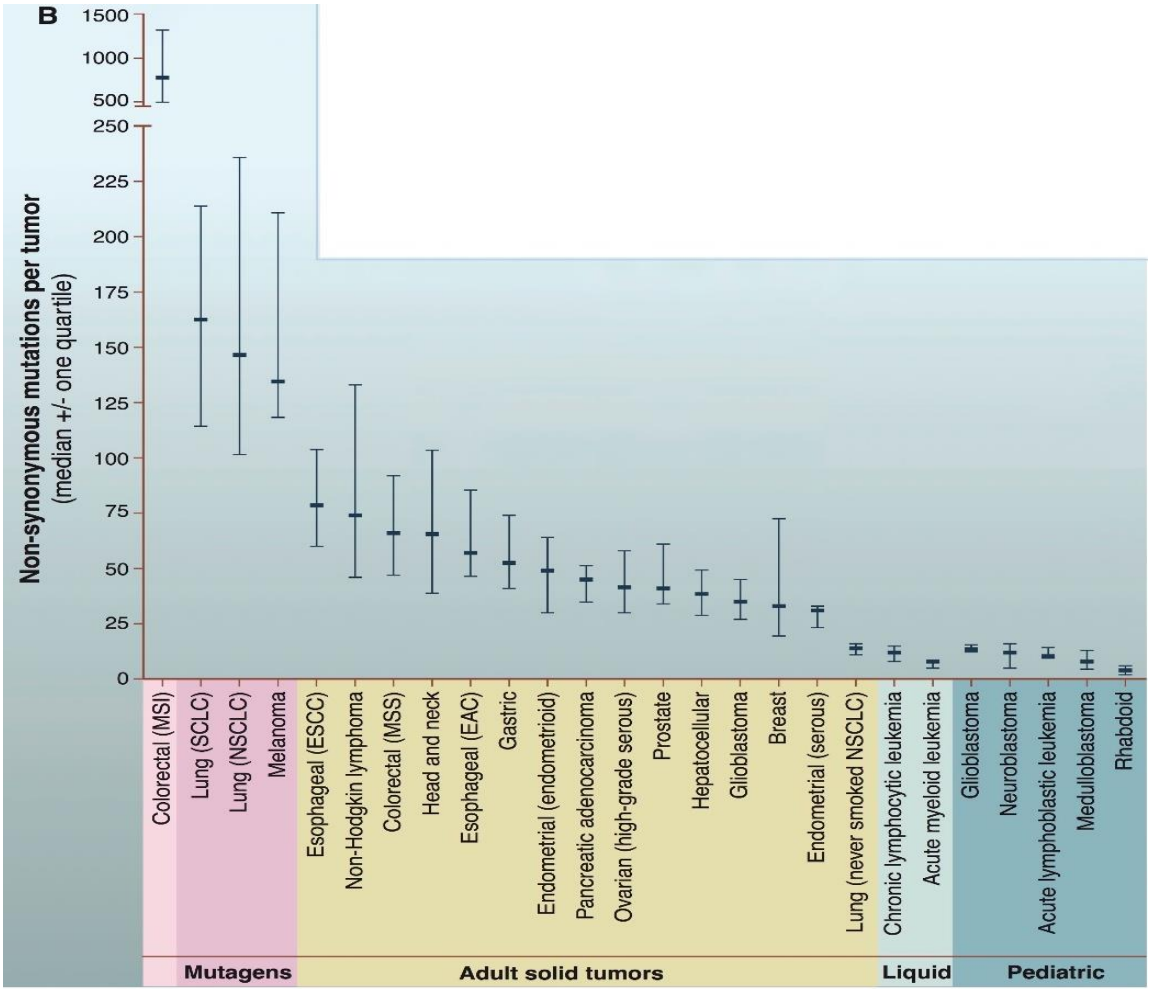

Fig. 7 ***Representation of non-synonymous mutations in individual tumor types.*** *The graph depicts tumors based on the average number of non-synonymous mutations detected in each individual tumor type from the highly to the scarcely mutated* (45).

Despite the existing lists of most commonly occurring mutations, there is still a variability present among individual tumors in terms of types and frequency of the present mutations. Mutations accumulated in tumor cells vary on four main levels; intratumoral, among the cells of one tumor, intermetastatic, among different metastatic lesions of the same patient, intrametastatic, among the cells of an individual metastasis and interpatient (45). The mutation rates range from 0.1 to 100/Mb across patients before repair (49). The variation among mutations is caused by many factors. The crucial factors, which increase the mutational load of tumor cells are the aspect of time, exposure of the tissue to mutagenic and inflammatory stimuli such as cigarette smoke, UV light (29, 50) and even microbiota (29, 51). UV radiation is significant for melanoma development (47), cigarette smoke for NSCLC and microbiota for CRC (29).

The temporal effect has been proved by higher numbers of mutations found in older patients compared to the figures acquired from pediatric patients. The significance of the time factor is also noticeable within the tumor mass itself, as the mutations continue to accumulate over time. Tumors evolve from premalignant benign lesions to malignant ones. This enables the researchers to determine where the tumor originated, based on tissue- and cell-of-origin site with the highest rate of mutations (45). The effect of time of replication of a DNA region during the cell cycle is also significant. It has been determined that the later the particular region is replicated the higher the possibility of arising mutation. This effect might be caused by the diminishing pool of nucleotides to choose from as the replication progresses. Potential for a mistake rises 2,9 fold in the latest percentile compared to the earliest percentile (49) of replicated bases. Another reason for a higher rate of mutations later in the cell cycle is that the later the mutation occurs, the less time there is to repair it.

For the purposes of T cell immune response, it is essential to focus on those mutations, which appear in the protein-coding regions and alter the presented epitope. Protein-coding regions represent only 1.5% of the total genome (45). Any genetic alteration, which affects a protein-coding region and can potentially lead to production of mutated peptides, is significant (8, 27, 52). Studies have been conducted in order to determine the proportion of non-synonymous somatic mutations capable of inducing an Ag- specific T cell immune response (50). According to the research one third (17) to a half (53) of the selected non-synonymous mutations is antigenic. This provides a large pool of candidate neoepitopes to be analyzed. Specific T cell immune response must nonetheless be confirmed to use the non-synonymous mutations as a biomarker.

Since the genomic alterations are essential for tumor development, numerous studies have been conducted in order to collect as much information about the features of the mutations as possible. To catalogue the genes responsible for the mutations leading to initiation and progression of tumor growth exome sequencing proved to be a robust method (24, 34, 49). Three groups of mutations relevant to T cell reactivity towards antigenome were identified at the core of tumor heterogeneity in chronic lymphocytic leukemia (CLL):

- missense mutations, caused by single base substitutions
- frameshift mutations
- splice-site mutations (8).

A general overview of the frequency of these types of mutations is presented in Figure 2 in the work by Rajasagi et al. (8). The missense mutations are the most abundant among oncological patients (8, 45). It is easy to verify their sequence by sequencing methods ensuring high confidence of their identification (46). Single-base substitutions present a great pool of exploitable neoepitopes to be targeted by reactive T cell clones.

Frameshift mutations and splice-site mutations are rare compared to missense point mutations. On the other hand, they can potentially generate longer sequences of unique CLL-specific amino acids (8) and be a source of neoepitopes (51). Researchers have established that there are notoriously mutated regions, which appear across a range of tumor types as shown in *Fig.8* (46). A comprehensive list of such mutations helps narrow down the candidate neoepitopes.

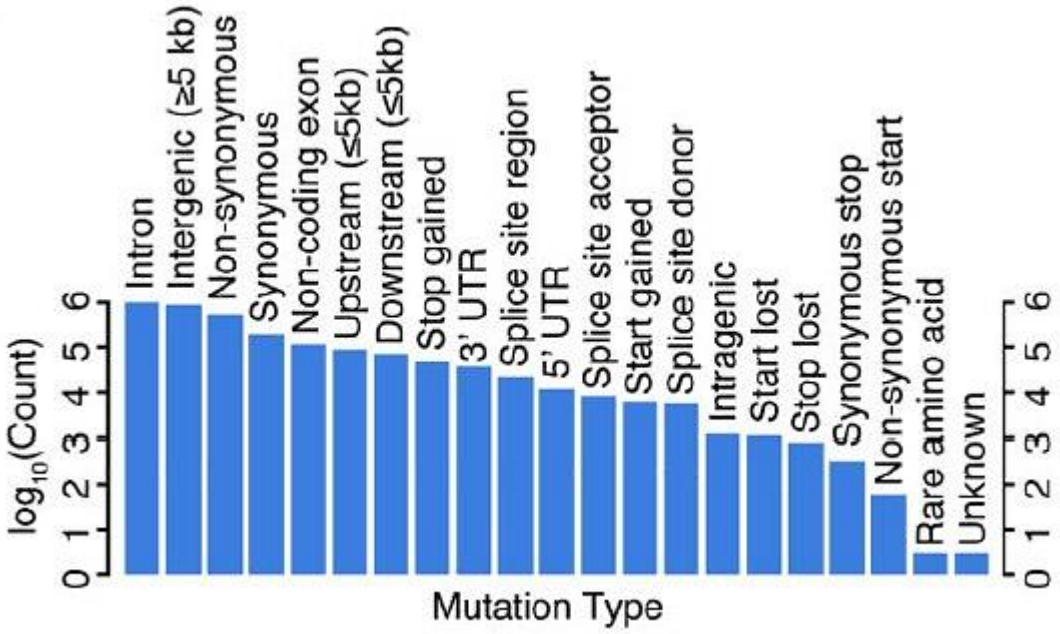

Fig. 8 ***Most commonly occurring mutations.*** *A graph depicting the typically occurring mutations on a logarithmic scale to show the frequencies of individual types of mutations in various tumor types. It is clear that introns are most frequently effected followed by intergenic alterations and then by non-synonymous mutations. For the T cell reactivity non-synonymous mutations are essential, leading to base substitutions. T cells recognize and target these delicate differences* (46).

3.1.2 Interpatient differences in mutations

A compilation of the most common types of mutations and their frequency in the most common types of tumors has been assembled (49) and is shown in *Fig.9.* Vast efforts have resulted in construction of four main databases considering both the genetics of tumor cells (54) and a comprehensive catalogue of neoepitopes and their MHC binding affinities suitable as targets for immunotherapy (17–19).

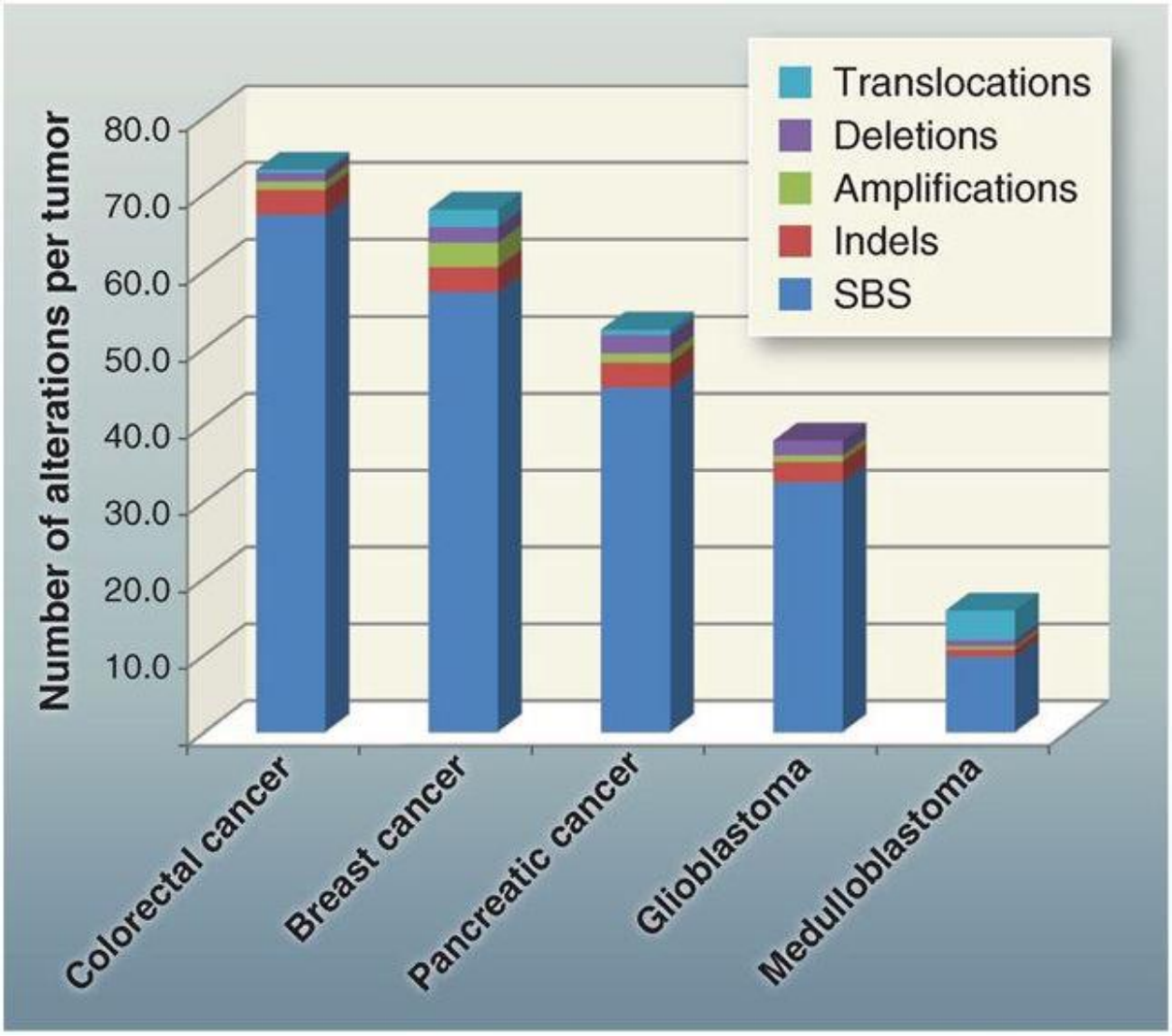

Fig.9 ***Types and rates of mutations in the most common tumors.*** *This graph presents the average number and type of genomic alterations detected in the most commonly occurring tumors. As evident from the bars, single-base substitutions are the most frequently present mutations across a range of tumors* (45).

The TRON Cell Line Portal (TCLP) database was constructed based on 1082 cancer cell lines. The research team used raw RNA-Seq data for the assessment of MHC molecules. They were assessed regarding type and binding prediction matched with annotated cell lines. The resulting database serves as a tool for predicting antigenic mutations as well as catalogue of predicted MHC class I and class II binding neoepitopes. A table of neoepitope catalogue for colon carcinoma is presented in Figure 4 in their paper (18). Similarly, a catalogue of tumor cell line immunologic information has been created. It also enables the prediction of antigenic mutation candidates for 108 commonly used cancer lines (17). These catalogues and databases are extremely useful in the effort to find a common neoepitope for a large cohort of patients.

Due to different exposure to external stimuli and genetic make-up of each patient the catalogues are not yet fine-tuned to predict the best potential candidate epitopes for each individual patient. Despite all the acquired knowledge it remains a challenge to prioritize the best candidate neoepitopes, which would induce the T cell response in a wide cohort of patients. This is caused by the high number of presented neoepitopes in each individual tumor. It is essential for the candidate mutant epitopes to be prioritized and more importantly tested.

3.2 Antigenome definition

The crucial term for understanding of the T cell reactivity towards arising tumor cells is the antigenome. Antigenome is a repertoire of all patient-specific tumor-associated epitopes presented to T cells by APC (6). In a broad sense, antigenome is a term used for peptides encoded in the genome of the tumor cells, which are eventually targets for the Ag specific receptors, BCR and TCR. For the purpose of this thesis the focus will be solely on the TCR. Important characteristic adding to the antigenicity of the arising epitopes is the altered protein folding of the mutated epitopes. This folding generally leads to reduced stability of the epitope, which increases the likelihood of APC presenting the neoepitope to the reactive T cell clone. Presentation of candidate neoepitope to TCR further increases the probability of inducing adaptive immune response (15). Precise identification of the candidate tumor-specific neoepitopes will serve as another in the line of predictive markers.

4 Antigenome – T cell reactivity

4.1 The role of T cells in tumor eradication

The two previous chapters have covered the nature of mutations present in tumor cells leading to development of tumor mass as well as the nature of T cell immune response. This chapter is focused on the mechanism of interaction between the T cells and the antigenome, which is crucial for tumor eradication. The research and application in therapy must face two major challenges. First specific neoepitopes are rarely shared between patients (32) and second tumors tend to evade the immune system. These challenges prove the necessary application of individualized approach to each individual patient in order to eradicate the tumor cells.

4.2 Interaction of T cells with neoepitopes

Clear identification of the antigenic neoepitopes with optimal binding affinity to MHC class I would lead to increase the number of tumor cells targeted by the T cell immune response. Tumor cells were derived from acute myeloid leukemia (AML), melanoma, RCC and lung-cancer (15). Each MHC molecule shows a different number of binding peptides and the so called facilitating mutations. The facilitating mutations are largely composed of peptides in which the mutated residue appears in a peripheral residue position (15). Furthermore, MHC molecules carrying bound self-epitopes can be easily obtained from serum of patients. This means that MHC complexes from solid tumors reach the bloodstream and can be recovered. It allows for development of multiplex tetramers enabling further study of the T cell antigenome interaction in a non-invasive way (16).

Recently an analysis has been developed that identifies antigenic neoepitopes and reactive T cell clones simultaneously (29). The research team developed a computer based method, which analyzed information from 9142 RNA-seq datasets assembled from 29 tumor types from The Cancer Genome Atlas (TCGA) and inferred their complementarity with T cell clones CDR3 sequences. CDR3 is the most variable region in TCR. CDR3 serves as a tool for T cell clone identification. The research team was able to identify three potential target neoepitopes, which can be exploited in patient prognosis (29).

4.3 Tumor evasion of the immune system

One of the major challenges in treating tumors is their ability to evade the immune system by creating escape clones. It is the main obstacle in targeted therapies because the tumors eventually develop an escape clone. The primary means of tumor escape mechanism is the loss of antigen expression (9). In cases when the tumor cells escape the immune system surveillance no specific $CD8^+$ T cells are detected in the patient´s serum, confirming the hypothesis that there were not antigens presented on MHC class I molecules for the CD8+ T cells to recognize and target (52). The significance of T cells in the process of tumor eradication is proved once again. The loss of antigen presentation is caused by epigenetic changes resulting in epigenetic silencing of antigen expression. Epigenetic silencing happens via DNA methylation, which was proved by the reverse epigenetic silencing inhibiting methylation (9). This way the T cells are incapacitated to perform their role in the eradication of tumor cells.

Other evasive mechanisms include depletion of immunosuppressive cells and upregulation of T cell immunoinhibitory molecules, such as PD-1 and LAG-3 in hypermutated tumors (55). Increase in immunosuppressive cells, myeloid-derived suppressor cells (MDSC) and regulatory T cells (T_{reg}) and downregulated immunoinhibitors and MHC I molecules were observed (56) in non-hypermutated tumors. However, immunosuppression by tumor cells leads to NK cell response and causes eradication of the tumor cell anyway. The tumor cells are also capable of employing already present mechanisms pathologically. One of such mechanisms is the exaggeration of normal regulatory circuits that control the self-tolerance (57).

Despite the mechanisms evolved to evade the immune system, when the T cells are reactive they are very effective in tumor eradication or at least in tumor growth stabilization. In patients with as few as one predicted mutation leading to expression of antigenic peptide, the risk of death has been significantly lowered, hazard ratio (HR)= 0.44 (28). The effectivity of T cell immune response has been proved. This feature can be exploited in personalized medicine.

5 Patient-oriented tumor treatment

5.1 Personalized treatment

The goal of today´s research is to design a treatment, which would target the tumor growth specifically in an individual patient while minimizing the adverse effects. Personalized approach would limit side effects, overtreatment and adverse effects which are associated with the currently used chemotherapy (58). Great advances in the prediction and detection methods have led to success in the application of personalized treatment. In the future each individual patient will be treated based on their own genetic make-up, the non-synonymous mutations present in the tumor cells and the state of their immune system at the time of treatment. Regarding the antigenome-T cell interaction the personalized treatment would target present tumor-specific neoepitopes with the pre-existent patient derived reactive T cell clones.

5.2 Patient stratification

In today´s clinical practice, the tumors are generally assessed using the so called TNM (tumor, nodes, metastasis) classification system. TNM system summarizes data on tumor burden (T), presence of tumor cells in draining lymph nodes of the region (N) and whether or not metastases have developed (M) (59). It is commonly applied to estimate prognosis and recommend suitable treatment. However, despite its widespread use, the TNM system does not predict patient´s response to therapy and does not provide information for patient stratification.

For the purpose of an appropriate therapy management and for better prognosis a method called Immunoscore has been designed. Immunoscore quantifies the *in situ* immune infiltrate and assesses the patient´s immune status. It is based on the identification of the type, functional orientation, density and location of adaptive immune cells within distinct tumor regions. Significant correlation between density of the immune cell populations and the patient´s clinical outcome has been found. Immunoscore takes into account the density of individual cell types, such as CD3+ lymphocytes and CD8+ cytotoxic T cells in the core of a tumor (60) and helps determine the patient´s prognosis. In a study of 602 tumors Immunoscore has proved to determine the prognosis accurately. The tumors were analyzed and measured for the CD45RO+ cells. Five years after diagnosis only 4,8% of patients with high densities of CD8+ CD45RO+ cells experienced CRC recurrence while 75% of patients with low densities suffered from CRC relapse (61). This particular example proves that Immunoscore has a great prognostic potential

aiding clinicians determine the right course of treatment and help them predict patient´s reaction to the applied treatment (60).

Even patients with the same characteristics, such as the same type of tumor, stage of tumor, metastases or age and gender present diverse reaction to treatment (59). To achieve as precise prediction as possible more aspects are currently evaluated in the patients. First one is the presence of PD-1 on the tumor cells, evaluated by pharmacokinetic analyses performed on collected blood samples. Patient blood samples are immunohistochemically tested for the presence of anti PD-L1 antibody. Tumor cells used in the study were from 207 patients. Patients suffered from various types of tumors: 75 from NSCLC, 55 from melanoma, 18 from CRC, 17 from RCC, 17 from ovarian cancer, 14 from pancreatic cancer, 7 from gastric cancer and 4 from breast cancer (38).

Patient stratification must be performed using a combination of TNM system and Immunoscore. Ideally the two systems will be complemented with personalized prediction of binding affinities of the present neoepitopes to the MHC molecules and the identification of corresponding reactive T cell clones (29).

5.3 Immunotherapy

Immunotherapy has been exploited in the treatment of tumors because it leads to durable tumor remission in responsive patients (11, 36). Immunotherapy is a form of treatment, which employs a great range of tactics using the T cells and antibodies of the adaptive immune system alongside cytotoxic properties of innate immune system (62). The goal of immunotherapy is to activate the immune system *en masse* (63). The activation of immune system prevents development of tumor escape clones, which is a side effect of targeted therapies. There are three main possibilities of immunotherapy application: vaccines, adoptive lymphocyte therapy and checkpoint inhibitors (62, 63). Therapies, which employ a wide range of cells such as checkpoint inhibitors unleashing the reactivity of T cell populations present an effective treatment plan. Nonetheless the response rates of patients profiting from the applied immunotherapy reach only 20%-25% (11). The stratification must be improved in order to identify the responsive patients more accurately and treat them accordingly.

5.3.1 The checkpoint inhibitors used in immunotherapy

Applying the checkpoint inhibitors is an excellent way of avoiding the tumor evasion of the immune system. The checkpoint inhibitors are used because the more reactive T cell clones interact with antigenic tumor neoepitopes the sooner they are exhausted. As a result, T cells are inhibited to protect the organism. Checkpoint inhibitors reverse the situation so that reactive T cell clones can target the tumor cells again. Because the checkpoint inhibitors activate the immune system as a whole they have a great potential in targeting the tumor cells. The types of tumors with higher mutational burden have a higher number of presented neoepitopes and therefore are more likely to be successfully targeted and eradicated by the antigen-specific T cell clones, *Fig.10*. Consequent neoepitope presentation on tumor cells leads to patient´s durable clinical benefit (11, 25, 34, 36, 39) as shown in *Fig.10*. Tumors with a high number of mutations are lung cancers and melanomas (64), renal-cell tumors (23, 35) and microsatellite instable (MSI) CRC (51). The number and diversity of reactive T cell clones is also important. Highest T cell clonotype diversity was observed in CRC, NSCLC, mesothelioma and melanoma (29). These tumors are suitable for checkpoint inhibitor therapy, because of the high number of pre-existing reactive T cell clones. Checkpoint inhibitor therapy includes anti PD-1 and anti CTLA-4 *(cytotoxic T lymphocyte antigen 4)* therapies, which were successful in the clinic (11).

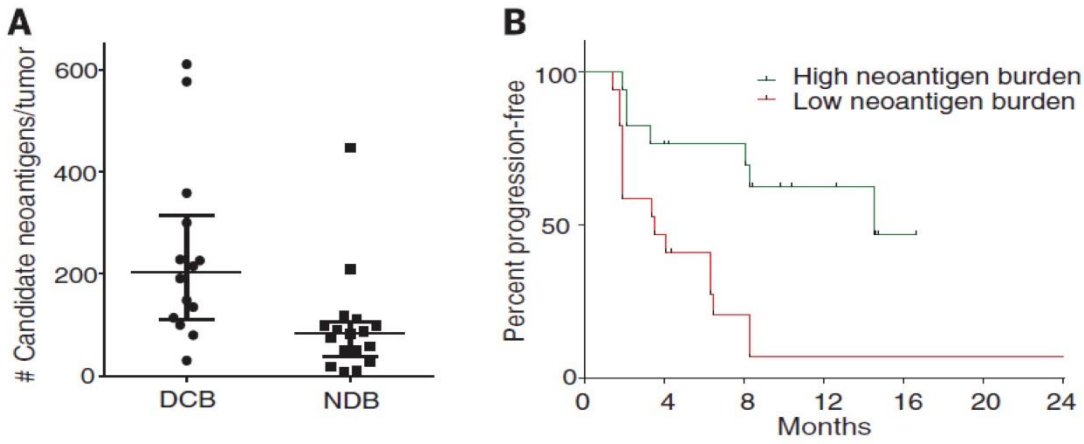

*Fig. 10 (A)**Neoepitope load in patients.** shows neoepitope (here termed neoantigen) load in patients with durable clinical benefit (DCB) compared to patients with no durable benefit (NDB). (B)**Progression free survival based on neoepitope burden.** The graph depicts the progression free survival of patients in months based on their neoepitope burden* (34).

The presence of checkpoint inhibitors, CTLA-4 and PD-1 on the T cell clones proves crucial in the search for efficient treatment (11). Interestingly, a strong correlation of the high mutational burden with effective application of the suppression of checkpoint inhibitors has been found (12, 51). The results in clinical trials were satisfying with the monoclonal antibody against CTLA-4 prolonged survival in treated melanoma patients (11). A combination of both anti-CTLA-4 and anti-PD-1 antibody shows promising durable regression of up to 2.5 years in patients with advanced melanoma (36).

Use of the method has proved successful in 18% of patients with NSCLC, 28% of melanoma patients and 27% of renal-cell tumor patients (37). Similar study conducted on melanoma patients reported 24% rate of objective responses in patients (36). In another study objective response was achieved with 29% of patients and additional 27% of patients showed stabilization of advanced RCC progression (35). In the mentioned studies numbers of patients responsive to the treatment differed according to the type of tumor but the rates of objective response fall between 20% and 25% (11). Checkpoint inhibitors also seem promising in the treatment of MSI CRC compared to the microsatellite stable (MSS) CRC. MSI CRC has a higher mutational burden, which makes it a better target for the checkpoint inhibitor therapy. Even though research showed that MSI CRC does not react very well to anti PD-1, high neoepitope burden and high levels of TIL different checkpoint inhibitors are being tested. Different checkpoint inhibitors are CTLA-4, LAG-3 and IDO *(indolamine 2,3-dioxygenase)* (51).

Despite the optimistic outcomes of the studies and the highly promising effectivity, immunotherapies frequently lead to development of adverse effects in patients. According to some studies as many as 91% (38) or even 93% (36) of treated patients may suffer from some grade of adverse effects as defined by the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. Among the most commonly present adverse effects were, skin related problems such as rash, pruritus and vitiligo, gastrointestinal disorder, endocrine disorder ranging from hypothyroidism to insufficiency and to autoimmune thyroiditis. In some patients development of autoimmune disorders was observed (11, 35–38). The adverse effects caused by the imposed treatment were mostly manageable and the treatment was not discontinued or only until the adverse effects were controlled.

Objective responses to application of solely anti-PD-1 drugs were also rapid and durable (11, 34, 65). In one study human monoclonal antibody, which specifically targets PD-L1 was applied.

In those patients, who responded well, researchers observed median of 18 week-long progression-free survival (65). Nonetheless only between 13% and 26% of patients responded to the treatment. The patient stratification is essential in order to determine which patients would benefit from immunotherapy (65). The immunotherapy has a great potential in oncological treatment. Researchers assume that the numbers of responsive patients will rise significantly after unleashing the Ag-specific T cell immune response targeting tumor neoepitopes (10, 11, 20, 25, 33, 35).

6 Conclusion

Tumor growth is one of the most common causes of death in developed countries. Therefore, it is essential to develop effective therapy, which would lead to durable remission and increased quality of patient´s life. Recently immunotherapy exploiting checkpoint inhibitors has experienced a great boom in its practical application in the clinic. Checkpoint inhibitor immunotherapy has achieved remarkable results in the responsive patients. Unfortunately, only around 25% of patients are responsive to the checkpoint inhibitors. The goal of research in oncology treatment using immunotherapy has been the stratification and right choice of well responding patients.

Responsive patients share specific traits that can be exploited as biomarkers, which facilitate the development of efficient patient stratification. These traits are pre-existence of reactive T cell clones and high mutational burden of the tumor mass. It has been established that pre-existence of T cell clones reactive to the specific neoepitopes is crucial for successful treatment. Additionally, presence of TIL, inhibitory molecules and potentially MHC class II molecules. Contrary to popular belief the higher the mutational burden of a tumor mass, the higher the chance that a reactive T cell clone exists and will target the tumor cell. This makes high mutational burden an excellent prognostic marker. Rapid development of new technologies allows for the prediction of candidate MHC class I binding neoepitopes which further increases the precision of the administered therapy. Lastly it is important to acknowledge the beneficial feature of T cell autoreactivity and employ the benefits of the process in the used therapy to ensure complete and durable tumor remission.

Modern technology presents the researchers with unprecedented possibilities in terms of identification of the tumor somatic mutations. The identification allows for specific targeting of the most antigenic neoepitopes. The combination of both predictive and detecting methods helps determine how to target the tumor cells more accurately. Treatment specifically targeting neoepitopes limits the adverse effects. In order to exploit the currently available methods to their fullest potential they must be combined. Patient stratification must be performed using a combination of TNM system and Immunoscore. Ideally the two systems will be complemented with personalized prediction of binding affinities of the present neoepitopes to the MHC molecules and the identification of corresponding reactive T cell clones.

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